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Protein identification using a nanoUHPLC-AP-MALDI MS/MS workflow with CID of multiply charged proteolytic peptides

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ABSTRACT

Liquid AP-MALDI can produce predominantly multiply charged ESI-like ions and stable durable analyte ion yields with samples allowing good shot-to-shot reproducibility and exhibiting self-healing properties during laser irradiation. In this study, LC-MALDI MS/MS workflows that utilize multiply charged ions are reported for the first time and compared with standard LC-ESI MS/MS for bottom-up proteomic analysis. The proposed method is compatible with trifluoroacetic acid as an LC ion pairing reagent and allows multiple MS/MS acquisitions of the LC-separated samples without substantial sample consumption. In addition, the method facilitates the storage of fully spotted MALDI target plates for months without significant sample degradation.

1. Introduction

Liquid chromatography (LC) coupled with mass spectrometry (MS) has become one of the most powerful analytical techniques for biomolecular studies. The development of electrospray ionization (ESI) [1] and the easy coupling of LC with ESI made it the preferred separation method for high-sensitivity MS analysis. Matrix-assisted laser desorption/ionization (MALDI) MS [2,3] has shown to be a valuable alternative to ESI often providing complementary information [4]. Compared to the on-line coupling of LC to ESI MS, LC is usually performed off-line when coupled to MALDI, depositing LC eluent fractions on a target plate before MALDI MS analysis [4–8].

Although on-line LC-ESI MS coupling is fast and straightforward, the off-line approach offers certain advantages by decoupling LC separation and MS analysis [9]. Firstly, LC separation and MS data acquisition can be independently optimized allowing the usage of additives in the LC runs which are not compatible with MS. Secondly, MS analysis can be performed without time constraints, enabling truly data-dependent acquisition (DDA) workflows. Finally, separated and MS-analyzed samples can be archived and revisited for subsequent re-analysis. These and other advantages have driven the development of various automated LC eluent fractionation/deposition systems [5,10–15] as well as advanced off-line MS acquisition workflows [7,9,16–18].

One of the challenges of conventional LC-MALDI coupling arises from the usage of solid crystalline samples for MALDI. Solid MALDI samples often provide poor sample homogeneity resulting in high ion yield variation which complicates the automation of MS data acquisition. In contrast, liquid MALDI samples not only offer better sample homogeneity [19,20] but also exhibit self-healing properties eliminating the need to adjust the laser desorption position during the acquisition, thus providing a substantially more robust system for automation [21,22].

The quality of mass spectral data is another important aspect, in particular for large-scale proteomics. ESI at atmospheric pressure (AP) effectively decouples mass measurement from ionization, facilitating hybrid mass spectrometry [23] and expanding the range of analytical tools available prior to mass analysis [24]. In contrast, conventional MALDI is typically performed at high vacuum, with the ion source directly coupled to an axial time-of-flight (TOF) mass analyzer, and the quality of mass spectral data greatly depends on complex ion manipulation and appropriate mass calibration which often complicates tandem mass spectrometry (MS/MS) and its outcome [7,25]. To alleviate these shortcomings MALDI has been

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employed at elevated pressures and hyphenated with other mass analyzers [26–32].

Another distinct feature of ESI compared to MALDI is its predominantly multiply charged ion yield which has certain advantages for MS analysis. Firstly, it enables electron-capture dissociation (ECD) [33] and electron-transfer dissociation (ETD) [34] fragmentation techniques capable of preserving labile post-translational modifications [35]. Secondly, it often improves the quality of collision-induced dissociation (CID) MS/MS data [36–38]. Thirdly, multiply charged ions are better suited for ion manipulation and transmission in radio frequency ion guides and low-cost mass spectrometers with a limited m/z range [39].

Recent developments in MALDI MS now allow the production of ESI-like predominantly multiply charged ions at atmospheric pressure from both solid [40] and liquid [41] samples at high sensitivity [42], thus promising the combination of some of the advantages from both MALDI and ESI. However, to our knowledge so far, no studies have been performed applying these methods to large-scale and/or automated proteomic analysis. Here, an initial design of an automated bottom-up proteomic workflow utilizing multiply charged ions obtained by liquid AP-MALDI, and its comparison to a conventional LC-ESI MS/MS workflow is described. Areas of future development for transforming the described approach into a practical analytical tool are highlighted.

2. Materials and methods

2.1. Consumables

Acetonitrile (ACN), trifluoroacetic acid (TFA), 2,5-dihydroxybenzoic acid (DHB) and glycerol were purchased from Sigma-Aldrich (Gillingham, UK). Formic acid (FA) was obtained from Greyhound (Birkenhead, UK). HPLC-grade water (H_2O) was purchased from Fisher Scientific (Loughborough, UK) and a tryptic bovine serum albumin (BSA) digest from Protea Biosciences (Morgantown, WV, USA).

2.2. UHPLC

LC separation for this study was carried out on a nanoACQUITY UPLC (Waters Ltd, Elstree, UK) system with a trap column (nanoACQUITY UPLC 2G-V/Mtrap 5 μm Symmetry; 20 mm length, 180 μm inner diameter (i.d.), 5 μm particle size; Waters Ltd) and a reversed-phase analytical column (ACQUITY UPLC Peptide BEH C18; 10k psi, 250 mm length, 75 μm i.d., 1.7 μm particle size, 130 Å pore size; Waters Ltd). Both FA and TFA were used as LC mobile phase additives. For the experiments with FA, solvent A was 0.1% FA in H_2O and solvent B was 0.1% FA in ACN. For the experiments with TFA, solvent A was 0.1% TFA in H_2O and solvent B was 0.1% TFA in ACN. A volume of 5 μL of a tryptic BSA digest diluted to a concentration of 1 pmol/μL in 0.1% FA was injected and analyzed using a flow rate of 300 nL/min with the following gradient: 3% B at 0 min, linearly increased to 40% B at 30 min, followed by 3 min washing at 85% B from 32 to 35 min.

2.3. MALDI sample deposition

For the LC-MALDI MS experiments, the liquid matrix solution was prepared by dissolving 25 mg of DHB in 70% ACN and adding 60% of glycerol by volume. A volume of 0.5 μL of this solution was spotted onto each sample position of a MALDI target plate and left drying at room temperature for 30 min. Subsequently, the UHPLC eluents were collected on top of the pre-spotted liquid matrices in 30-s fractions (150 nL) by manual submerging the LC outlet capillary into the pre-spotted matrix droplet. A total of 60 fractions were prepared for each LC run. Finally, 0.5 μL of a 10-μM bradykinin solution was spotted to one of the remaining sample wells with pre-spotted liquid matrix for mass measurement accuracy checks. All MALDI samples remained liquid over the entire timespan of the experiment.

2.4. MALDI MS

For all MALDI MS experiments a Synapt G2-Si mass spectrometer (Waters Corporation, Wilmslow, UK) was employed. An in-house developed AP-MALDI ion source described in detail elsewhere [42] was optimized for the generation of multiply charged ions from liquid MALDI samples (see Fig. 1). Briefly, a commercial ESI source (Waters) for the Synapt G2-Si mass spectrometer was modified to accommodate a standard Waters MALDI target plate mounted on a PC-controllable XY-translational stage (Zaber T-LSM050A; Laser2000, Huntingdon, UK). The ion source's AP-to-vacuum interface was equipped with an additional resistively heated ion transfer tube in front of the vacuum inlet with a controllable gas flow regime which was operated at 25 W heating power and 180 L/h counter flow (~350 °C ion transfer tube wall temperature). The distance between the ion transfer tube and the MALDI target plate was 3 mm, and a 4 kV potential was applied to the target plate. A pulsed nitrogen laser (MNL 103 LD; LTB Lasertechnik GmbH, Berlin, Germany) with a pulse duration of 3 ns and a wavelength of 337 nm was used to irradiate the sample. The laser was operated at a repetition rate of 30 Hz and focused to a spot size diameter of ~150 μm with the laser pulse energy attenuated to 30 μJ. Please note that local H&S requirements, e.g. regarding laser safety and risk assessments, have to be satisfied as it was the case for this study.

MALDI MS data were acquired in ESI MS mode with ion mobility separation (IMS) enabled. The instrument settings controlling the nitrogen gas flow in the IMS cell, helium gas flow in the helium cell and argon gas flow in the trap cell were set to 90 mL/min, 180 mL/min and 2 mL/min, respectively. The drift time in the IMS cell was recorded with the 'IMS Wave Velocity' set to 650 m/s and 'IMS Wave Height' set to 40.0 V. A standard ESI MS survey method ('Mobility Fast DDA') was adapted for automated data acquisition. The MS survey scan duration was set to 1 s with a signal intensity above 100 triggering subsequent MS/MS acquisitions of 5 s. CID was carried out after IMS separation in a transfer cell using a collision energy ramp with the following settings: LM CE ramp start = 30, LM CE ramp End = 40, HM CE ramp start = 40, HM CE ramp End = 55. Singly charged ions were eliminated from the precursor ion selection list. The m/z data acquisition range was set to 100–2000.

For controlling the MALDI target plate XY stage movement, a Waters Research Enabled Software (WREnS; Waters Corporation) compatible script was developed. Prior to the start of the MS data acquisition the laser focus was positioned on the first sample and the laser was turned on. Within seconds MS data acquisition was manually started, which automatically triggered the target plate movement allowing consecutive stationary irradiation of each MALDI sample for 60 s before automatically moving to the next sample. Between each sample the XY stage moved the target plate to a blank sample spot for 5 s.

2.5. LC-ESI MS

For the comparative LC-ESI MS experiments the nanoUHPLC system was coupled to the Synapt G2-Si mass spectrometer using the Universal Nano Flow Sprayer (Waters) with a PicoTip emitter (20 μm; New Objective, Woburn, MA). IMS separation for these experiments was turned off. Similar to the MALDI MS data acquisition, a fast DDA method was used for the ESI MS data acquisition. The MS survey scan duration was set to 0.2 s with a signal intensity above 5000 triggering subsequent MS/MS acquisitions. The

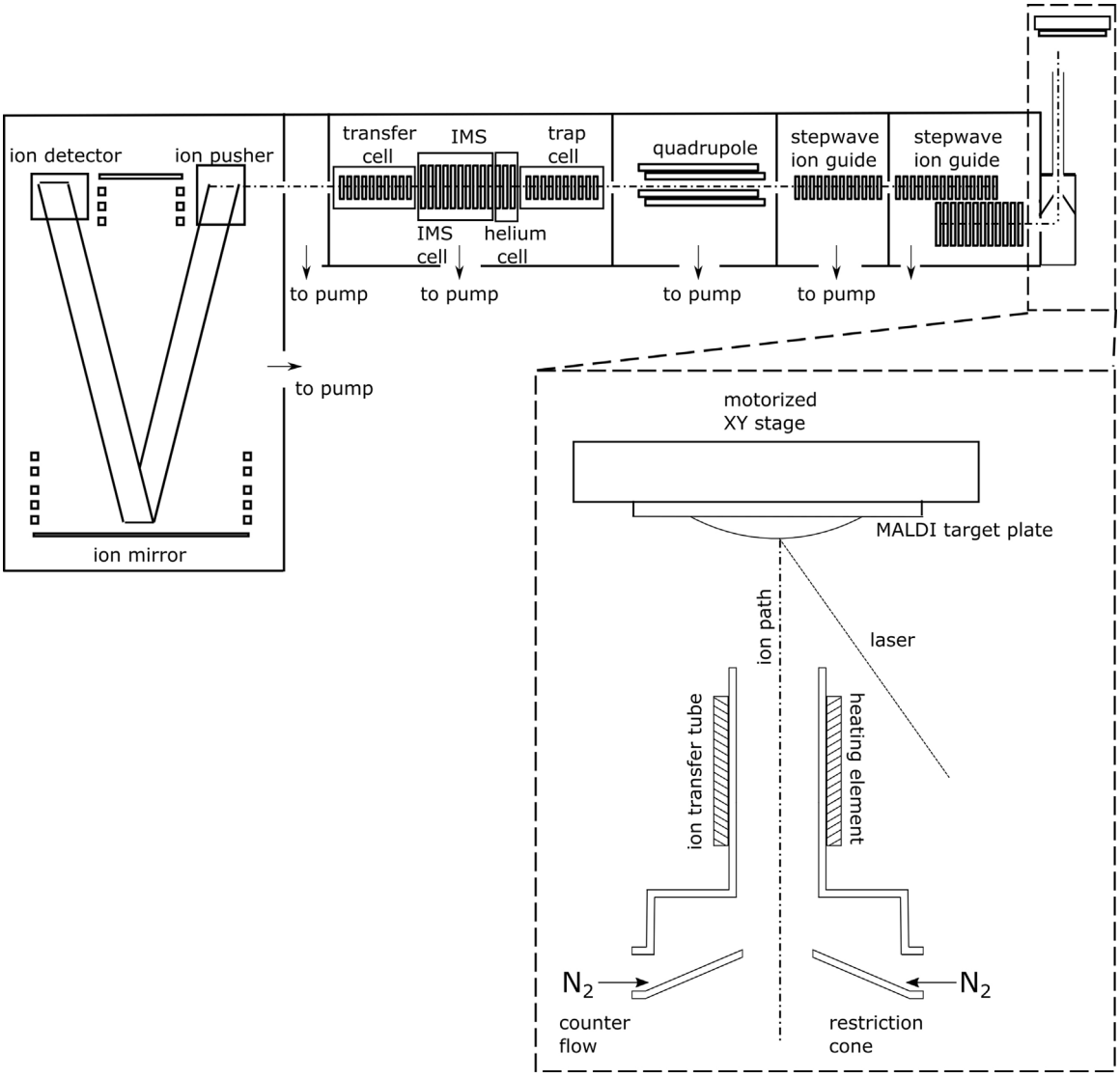

Fig. 1. Schematic of the Synapt G2-Si mass spectrometer with the in-house developed AP-MALDI ion source.

maximum allowed number of MS/MS acquisitions was set to 15 and the MS/MS scan duration was set to 0.1 s. CID was carried out in the trap cell using a collision energy ramp with the following settings: LM CE ramp start = 25, LM CE ramp End = 35, HM CE ramp start = 35, HM CE ramp End = 50. As for LC-MALDI MS, the *m*/*z* data acquisition range was set to 100–2000.

2.6. Data analysis

All raw MS data were submitted to Mascot Distiller (Version 2.3.2; Matrix Science, London, UK) for data processing and peak lists generation. The peak lists were then searched against the SwissProt database (version 2016_02; 550,552 sequences; 196,472,675 residues) using the MS/MS ion search routine of the search engine Mascot 2.4 (Matrix Science). The search parameters were 50 ppm for peptide mass tolerance, 2 for peptide charge state, trypsin for enzyme, carbamidomethyl (C) for fixed modifications, oxidation (M) and amidated (Protein C-term) for variable modifications, and 2 for max missed cleavages. The acquired data were self-calibrated based on the systematic mass error obtained from the Mascot search results using the 'Modify Calibration' method within MassLynx software (version 4.1; Waters). Doubly charged ion signals from co-analyzed bradykinin samples were used to validate the final calibration. The calibrated data were then resubmitted to the Mascot search engine.

For LC-ESI MS ion signal intensity analysis, Mascot search results were exported. The precursor ion intensity was extracted for each MS data query that led to the identification of a tryptic BSA peptide. If a peptide was identified in multiple queries the corresponding ion signal intensities were all summed up and used as a single data point.

3. Results and discussion

3.1. Proof of concept

The objective of the study was to demonstrate and evaluate LC-MALDI MS for bottom-up proteomics based on protein identification analysis using AP-MALDI MS/MS data obtained from CID of multiply charged proteolytic peptides. Due to the ability of liquid MALDI to generate robust and intense ion signals of multiply charged peptides, ESI MS/MS data acquisition can be mimicked and similar DDA routines can be exploited, resulting in MALDI MS/MS fragmentation data of multiply charged peptides for standard protein identification by readily available database search engines without any additional data processing steps.

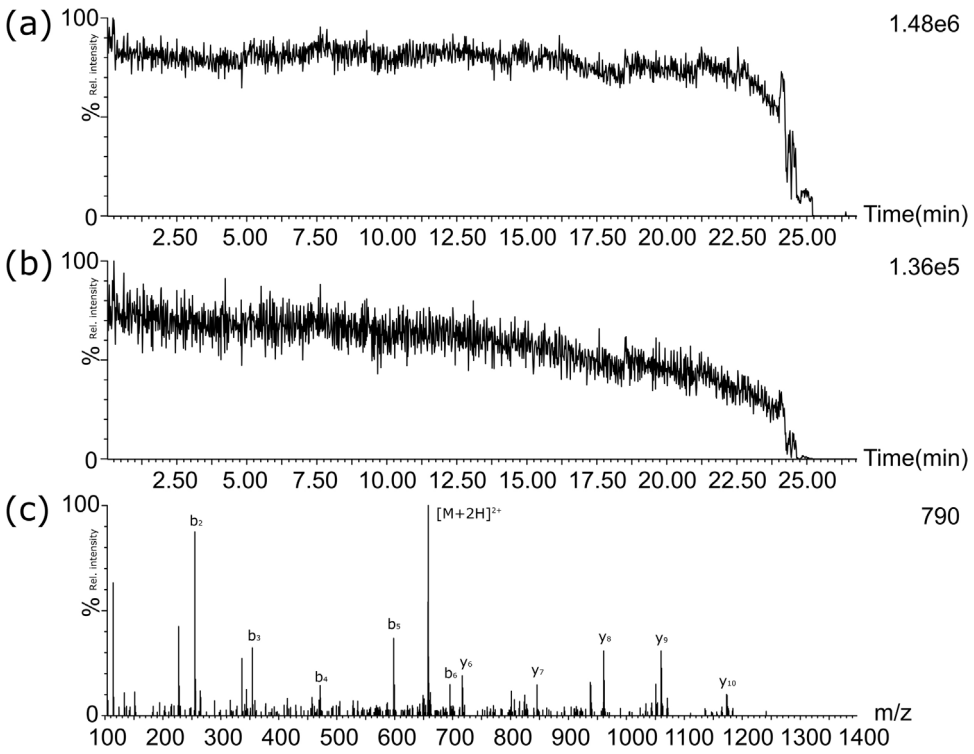

Fig. 2. AP-MALDI MS(/MS) data of a single liquid MALDI sample containing a nanoUHPLC fraction of 5 pmol of a BSA tryptic digest. The sample was already subjected to a total of ~10,000 laser shots in a previous analysis and re-analyzed directly from the MALDI target plate that was stored for 8 weeks. After the acquisition the sample was completely depleted by the laser irradiation using a repetition rate of 30 Hz and a laser energy per shot of 30 μJ. (a) Total ion chromatogram (TIC) over 25 min. (b) Extracted ion chromatogram (EIC) over 25 min with an *m/z* window of 652.6–655.6. (c) MS/MS spectrum of the BSA tryptic peptide HLVDEPQNLIK at *m/z* 653 acquired for 1 s.

For this study, a few optimization steps were performed to adapt the instrument manufacturer's standard ESI DDA routine to AP-MALDI ion generation. To promote greater sample/analyte desorption the laser repetition rate was set to its maximum value of 30 Hz and the laser pulse energy was raised from 16 μJ to 30 μJ. It is important to remind the reader that stable multiply charged peptide ion signals can be easily obtained by liquid MALDI from low femtomole amounts for tens of minutes or hours, while the same amount analyzed by nanoESI (e.g. at 300 nL/min), even when highly diluted in volumes of a few microliters, would only provide ion signals for a few minutes. Furthermore, the vast majority of a specific peptide typically elutes within 10–30 s using standard (nanoU)HPLC and is entirely consumed by (nanoU)HPLC-ESI MS analysis within this time frame. For the nanoUHPLC-MALDI MS measurements presented here, the eluted peptide amount is at best entirely collected in one fraction, i.e. one MALDI sample, of which only a small fraction is consumed by the subsequent MS analysis. Fig. 2 shows the stable long-lasting peptide ion yield achievable with liquid MALDI using a fraction of 30 s of a nanoUHPLC-separated BSA digest that had already been analyzed once before.

Although the sample had been stored for more than 8 weeks in a freezer after a first MS/MS measurement, only 1 s was needed for obtaining sufficient precursor ion signal for a quality MS/MS spectrum and the signal still lasted for almost 25 min at a laser repetition rate of 30 Hz, i.e. ~45,000 laser shots.

It was also found that by enabling the recording of ion drift times in the IMS cell the detected ion signal can be improved. With IMS turned on, different potentials are applied to the instrument's ion transport guide and additional gases are infused into the trap, helium, IMS and transfer cells of the instrument (see Fig. 1). Enabling IMS in liquid MALDI experiments led to a 20–100% signal increase. Therefore, this setting was applied to all MALDI MS measurements. In addition, IMS separation is also beneficial as it eliminates singly charged MALDI chemical noise ions from the survey scan.

In general, both MS and the corresponding MS/MS scan durations were set to their maximum software values of 1 s and 5 s, respectively, to compensate the above discussed difference in the detection of the potential analyte amount compared to ESI. Peptides with charge states of 2–4 were picked in the survey scans for subsequent MS/MS analysis.

To test these quick and easy optimizations for off-line LC-MALDI MS/MS proteomic analysis on an instrument optimized for ESI MS, an amount of 5 pmol of a tryptic BSA digest was loaded on a nanoUHPLC system with 0.1% FA as a mobile phase additive. The LC-MALDI MS analysis was performed as described above. Fig. 3 shows the base peak ion chromatogram and MS/MS spectrum obtained in this experiment.

All MALDI MS measurements were performed without lock mass data acquisition but the collected raw data were mass-corrected to eliminate systematic mass off-set errors (see Section 2.6). The data were then submitted to a Mascot search engine searching the well-curated SwissProt protein sequence database and resulting in 37 unique BSA peptide identifications with a 60% protein sequence coverage and a Mascot score of 626. The list of identified peptides and corresponding precursor ions is shown in Table 1. The average mass accuracy for these experiments was 5 ppm.

3.2. Sample archiving and additional peptide identifications by sample re-analysis

An advantage of liquid (AP-)MALDI is the extremely low amount of sample consumed for MS analysis. Only 10–30 laser shots, consuming <1 nL of a 1-μL sample droplet, are needed to obtain

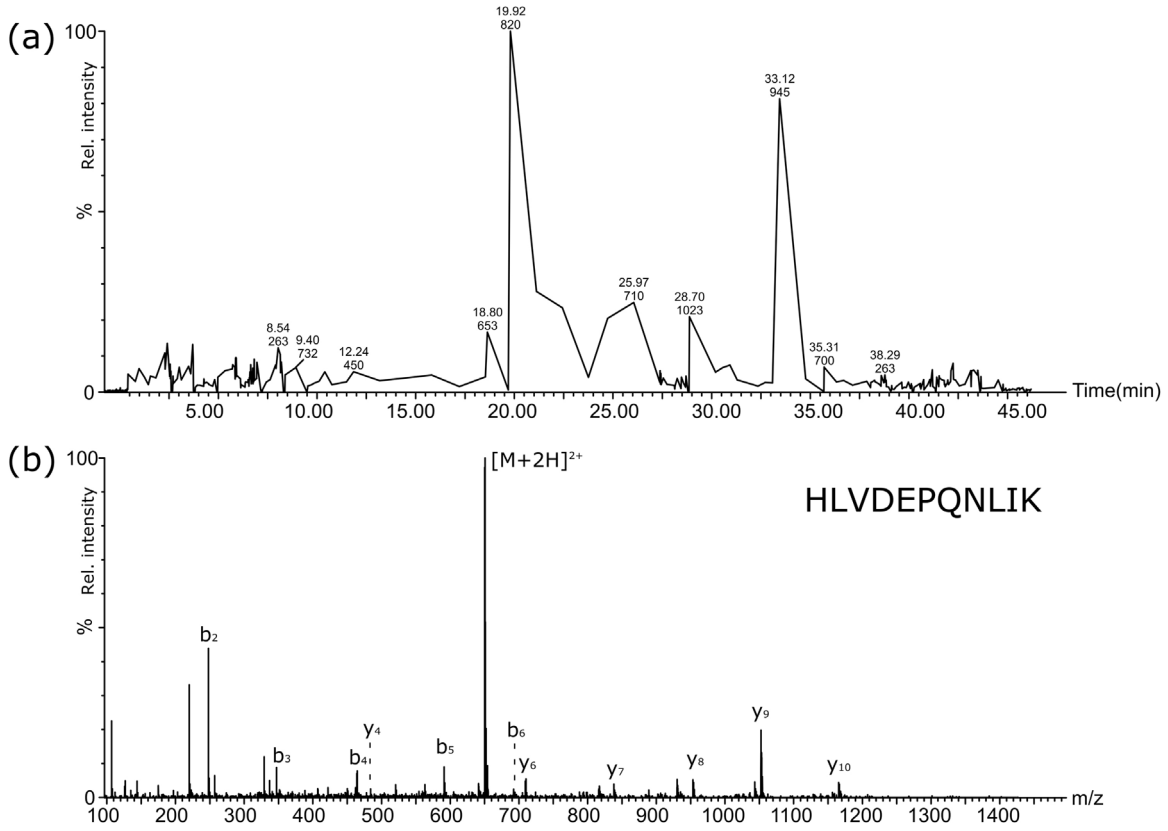

Fig. 3. a) LC-AP-MALDI MS base peak ion chromatogram of the analysis of 5 pmol of a tryptic BSA digest separated by nanoUHPLC. The chromatogram reflects the off-line MS data acquisition using a DDA routine, in which the time intervals between MS survey scans depend on the number of subsequent data-dependent MS/MS scans and can therefore differ substantially. b) AP-MALDI MS/MS mass spectrum of the doubly charged tryptic BSA peptide HLVDEPQNLIK (m/z 653) acquired at 18.8 min for 5 s.

sufficient analyte ion signal for MS/MS (see Fig. 2). The time required for such an acquisition is ≤1 s and should be even faster with high-repetition rate lasers. Moreover, initial single peptide experiments showed that spotted MALDI samples do not degrade significantly for at least up to two weeks (data not shown). Arguably due to the glycerol in the MALDI sample, droplets remain liquid during storage at −20 °C, thus avoiding freeze/thaw cycles which could potentially cause sample degradation. These properties make liquid MALDI attractive for applications where sample storage and re-analysis are required.

Whether complex peptide samples can also be stored for re-analysis for prolonged time periods without degradation was tested on one of the previously analyzed BSA digest samples. For this, an amount of 5 pmol of a BSA digest was LC-fractionated, spotted on a target plate and analyzed by liquid AP-MALDI MS/MS on the same day according to the protocols described earlier. The target plate with the spotted MALDI samples was then stored for 8 weeks at −20 °C for re-analysis. After the target plate was taken out of the freezer it was allowed to warm up to room temperature for 10–20 min and subjected to an additional five full MS(/MS) data acquisitions. During these additional measurements, images of a representative sample well were taken and are shown in Fig. 4. It is evident from these images that no visible shrinkage of the droplet size was observable during the experiment.

The acquired MS data were submitted to Mascot and the results obtained from the database searching are shown in Table 2. Overall, the experiment revealed no substantial sample degradation due to storage of 8 weeks at −20 °C or multiple re-analyses. Both images of the MALDI sample and the MS data suggest that during each AP-MALDI MS/MS acquisition extremely low amounts of sample are consumed. By combining all of the data sets, a total sequence coverage of 67% was achieved from a single LC run with 39 unique peptides identified in at least 2 MS data acquisitions (see Supplemental Table 1). For this experiment the LC mobile phase contained 0.1% TFA which is further investigated and discussed in the following section.

3.3. *TFA as LC mobile phase additive is compatible with liquid MALDI MS*

TFA is commonly used as an ion pairing reagent in HPLC to improve separation of peptides and proteins. In ESI, TFA is known to exert suppression effects on both peptide and protein ion signals arguably due to gas phase ion pairing and surface tension modification [43,44]. To test whether TFA causes similar ion suppression effects in liquid LC-MALDI, experiments were performed where FA in the LC mobile phase was substituted with TFA. The re-analyses experiment described in Table 2 (using 0.1% TFA as LC mobile phase additive) was repeated with 0.1% FA as LC mobile phase additive. The experiment using FA resulted in a total BSA sequence coverage of 64% from a single LC run with 38 unique peptides identified in at least 2 MS data acquisitions while it was 67% sequence coverage and 39 unique peptides for the experiment using TFA (see Supplemental Table 1). The comparison of these two experiments also shows that 35 unique peptides are common in both experiments. The sequence coverages achieved in these experiments are shown in Fig. 5.

The acquired data reveal that there are no adverse effects using TFA at the nanoUHPLC separation step. Two explanations are possible for such an observation. Firstly, prior to MS analysis there is sufficient time for TFA to evaporate from the sample droplet, thus resulting in a TFA-free sample. Secondly, because only a thin (top)

Table 1
List of BSA peptides identified in a single LC-AP-MALDI MS/MS experiment based on multiply charged precursor ions[a].

Observed *m/z*	Mass accuracy (ppm)	Charge state	Assigned peptide sequence	Score
625.3158	2.62	2	FKDLGEEHFK	35
582.3239	8.58	2	LVNELTEFAK	61
732.2951	−4.17	2	TCVADESHAGCEK	66
710.3494	−1.53	2	SLHTLFGDELCK	59
517.7398	−0.073	2	NECFLSHK	25
634.6274	−1.08	3	NECFLSHKDDSPDLPK	13
788.8845	−3.77	2	LKPDPNTLCDEFK	16
464.2555	11.1	2	YLYEIAR	23
1023.0143	−3.22	2	RHPYFYAPELLYYANK	7
682.3511	5.35	3	RHPYFYAPELLYYANK	17
944.9657	−1.41	2	HPYFYAPELLYYANK	85
874.3507	−6.26	2	YNGVFQECCQAEDK	51
379.7203	13.7	2	GACLLPK	7
461.7500	5.11	2	AEFVEVTK	15
395.2427	8.21	2	LVTDLTK	11
875.3266	−9.48	2	ECCHGDLLECADDR	10
722.3243	−0.46	2	YICDNQDTISSK	65
766.8898	−5.77	2	LKECCDKPLLEK	27
646.3018	−4.37	2	ECCDKPLLEK	12
878.6723	−1.25	4	SHCIAEVEKDAIPENLPPLTADFAEDKDVCK	24
820.0625	−3.07	3	DAIPENLPPLTADFAEDKDVCK	9
720.4090	−0.74	2	RHPEYAVSVLLR	35
480.6094	1.25	2	RHPEYAVSVLLR	20
642.3602	1.98	2	HPEYAVSVLLR	64
751.8116	1.50	2	EYEATLEECCAK	54
777.8296	−0.62	2	DDPHACYSTVFDK	25
653.3632	2.36	2	HLVDEPQNLIK	59
740.4035	2.85	2	LGEYGFQNALIVR	74
820.4689	−4.41	2	KVPQVSTPTLVEVSR	84
756.4206	−5.85	2	VPQVSTPTLVEVSR	43
449.7448	0.84	2	LCVLHEK	4
569.7560	6.02	2	CCTESLVNR	47
940.9591	−5.38	2	RPCFSALTPDETYVPK	29
627.6467	2.42	3	RPCFSALTPDETYVPK	12
954.4651	1.12	2	LFTFHADICTLPDTEK	4
571.8631	4.03	2	KQTALVELLK	36
507.8166	6.52	2	QTALVELLK	53
700.3503	0.44	2	TVMENFVAFVDK	57
708.3576	14.4	2	TVMENFVAFVDK + Oxidation	41
643.2731	3.43	3	CCAADDKEACFAVEGPK	1
554.2620	2.54	2	EACFAVEGPK	27

[a] The data were obtained from SwissProt database searching of the raw MS(/MS) data acquired from 5 pmol of a tryptic BSA digest that was factionated by nanoUHPLC with 0.1% FA in the mobile phase.

layer of the sample droplet is desorbed, possible electrophoretic separation of the TFA anions from the peptide cations caused by the applied voltage potential prevents their simultaneous desorption. The former has been confirmed by acquiring AP-MALDI mass spectra in negative ion mode using the same MALDI matrix and 0.2% TFA as analyte solution. Spectra were recorded immediately after sample spotting and after 30 min of sample drying at ambient pressure. After drying, the TFA deprotonated anion signal intensity dropped by ~70%. Interestingly, TFA evaporation from a sample droplet can also be registered by irradiating a TFA-free adjacent sample droplet without irradiating the TFA-containing sample droplet. It can be speculated that the TFA anions are produced by evaporation and gas-phase proton transfer or a secondary electrospray ionization process of fused aerosols [45].

3.4. Comparison to LC-ESI MS

In a final comparison, the same BSA digest analyzed by LC-MALDI MS/MS was also analyzed using a standard LC-ESI MS/MS DDA workflow with 0.1% FA in the LC mobile phase and no IMS separation. Here, IMS was switched off due to concerns of detector saturation. This resulted in 66 unique peptide identifications and 77% sequence coverage. All peptides identified by liquid AP-MALDI MS coupled to off-line nanoHPLC separation were also found in the on-line LC-ESI MS data (see Supplemental Table 1). The difference in sequence coverage obtained by LC-MALDI MS and LC-ESI MS is shown in Fig. 6.

In general, the comparison between the off-line LC-MALDI and on-line LC-ESI analyses shows that the MALDI-generated peptides are also found in ESI MS. There is currently a lower number of tryptic peptides detected by MALDI MS. However, as a commercial instrument optimized for ESI data acquisition was employed for the measurements, and more importantly, only little advantage was taken from the fact that the sample consumption is roughly one order of magnitude lower, there is great potential to improve its sensitivity for proteomic workflows substantially. Fig. 7 shows that the difference in ESI signal intensity for the peptides detected by both methods compared to the ones detected only by ESI MS

Table 2
Database search results of AP-MALDI MS/MS re-analyses of the same liquid MALDI samples of an LC-fractionated BSA digest (5 pmol).

MS acquisition number	Storage time at −20 °C	Number of unique peptides identified	BSA sequence coverage (%)	Mascot score
1	none	37	57	761
2	8 weeks	37	58	1181
3	8 weeks	38	61	965
4	8 weeks	37	59	1177
5	8 weeks	37	59	1084
6	8 weeks	35	56	923

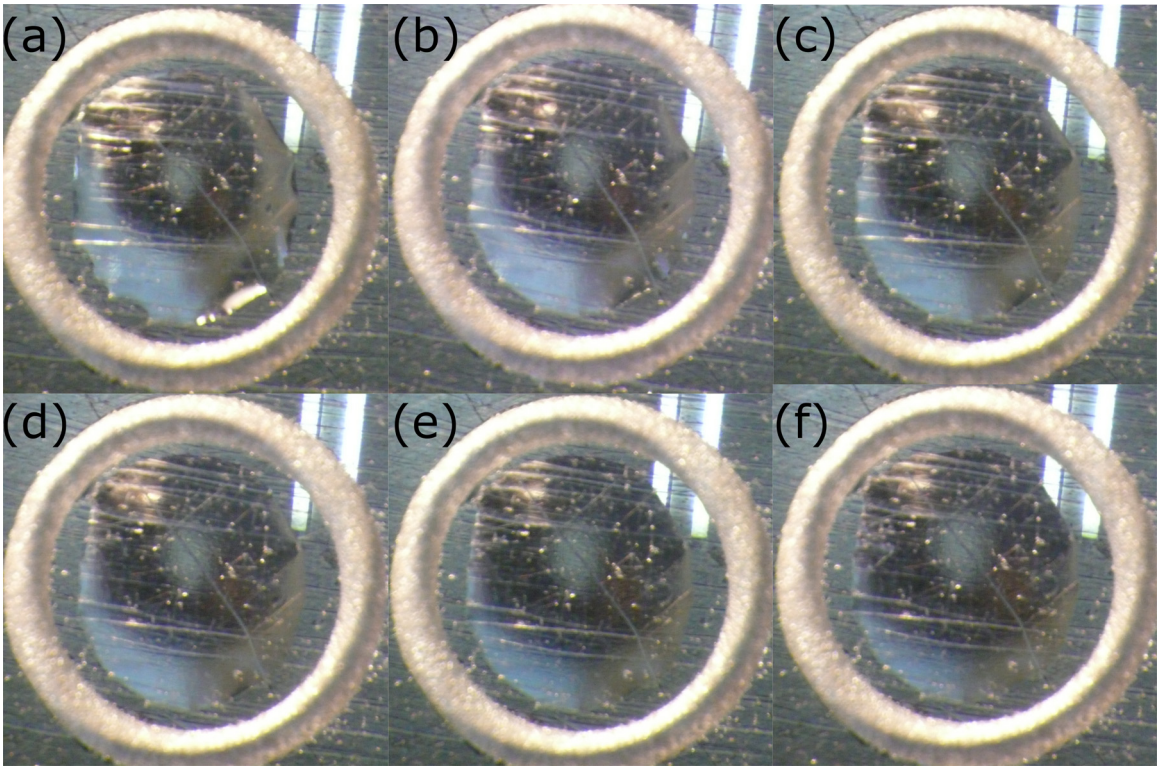

Fig. 4. Images of the same liquid MALDI sample taken during a set of 5 consecutive AP-MALDI MS/MS data acquisitions. Image (a) was taken before the first measurement, images (b–f) were taken after each AP-MALDI MS/MS data acquisition. During each of these measurements the sample was irradiated for 60 s with a laser repetition rate set to 30 Hz and a laser pulse energy of 30 μJ.

MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHR**FKDLGE** **EHFK**GLVLIA
FSQYLQQCPF DEHVK**LVNEL** **TEFAKTCVAD** **ESHAGCEKSL** **HTLFGDELCK**
VASLR***ETYGD*** ***MADCCEK***QEP ER**NECFLSHK** **DDSPDLPKLK** **PDPNTLCDEF**
KADEKKFWGK **YLYEIARRHP** **YFYAPELLYY** **ANKYNGVFQE** **CCQAEDK**GAC
LLPKIETMRE KVLASSARQR LRCASIQKFG ERALKAWSVA RLSQKFPK**AE**
FVEVTKLVTD LTKVHK**ECCH** **GDLLECADDR** ADLAK**YICDN** **QDTISSKLKE**
CCDKPLLEKS **HCIAEVEKDA** **IPENLPPLTA** **DFAEDKDVCK** NYQEAKDAFL
GSFLYEYSR**R** **HPEYAVSVLL** **R**LAK**EYEATL** **EECCAKDDPH** **ACYSTVFDK**L
K**HLVDEPQNL** **IK**QNCDQFEK **LGEYGFQNAL** **IVR**YTR**KVPQ** **VSTPTLVEVS**
RSLGKVGTR***C*** ***CTKPESER***MP CTEDYLSLIL NR**LCVLHEK**T PVSEKVTK**CC**
TESLVNRRPC **FSALTPDETY** **VPK**AFDEK***LF*** ***TFHADICTLP*** ***DTEK***QIK**KQT**
ALVELLKHKP KATEEQLK**TV** **MENFVAFVDK** **CCAADDKEAC** **FAVEGPK**LVV
STQTALA

Fig. 5. BSA sequence coverage obtained by two sets of LC-AP-MALDI MS/MS analyses of 5 pmol of a tryptic digest using FA and TFA, respectively, in the LC mobile phase. Sequences in **bold** were identified in both experiments in at least two MS/(MS) acquisitions. Sequences in *italics* were exclusively identified in the experiment with TFA in the LC mobile phase in at least two MS/(MS) acquisitions. Underlined sequences were identified only in the experiment with FA in the LC mobile phase in at least two MS(/MS) acquisitions.

is around one order of magnitude. These data are in agreement with the hypothesis that liquid AP-MALDI and ESI share the same ionization mechanism but the current limits of detection in liquid AP-MALDI MS result in a smaller number of identified peptides. Thus, relatively simple changes such as a higher laser shot repetition rate, optimized off-line collection of possibly smaller LC fractions and the introduction of droplet shrinkage, leading to smaller samples but higher analyte concentration as previously demonstrated [21], should all improve the detection limits in future MS-based proteomic workflows using liquid MALDI.

Data supporting the results reported in this paper are openly available from the University of Reading Research Data Archive at http://dx.doi.org/10.17864/1947.74

4. Conclusions

This is the first time that off-line LC coupled to (liquid) AP-MALDI has been shown to allow protein identification based entirely on the MS/MS analysis of multiply charged precursor ions. Simple adaptations of a standard ESI-based bottom-up proteomic workflow have

MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHR**FKDLGE** **EHFK**GLVLIA
FSQYLQQCPF DEHVK**LVNEL** **TEFAKTCVAD** **ESHAGCEKSL** **HTLFGDELCK**
VASLR**ETYGD** **MADCCEKQEP** **ERNECFLSHK** **DDSPDLPKLK** **PDPNTLCDEF**
KADEK*KFWGK* **YLYEIARRHP** **YFYAPELLYY** **ANKYNGVFQE** **CCQAEDKGAC**
LLPKIETMRE KVLASSARQR LRCASIQKFG ERALKAWSVA RLSQKFPK**AE**
FVEVTKLVTD **LTK***VHK***ECCH** **GDLLECADDR** **ADLAKYICDN** **QDTISSKLKE**
CCDKPLLEKS **HCIAEVEKDA** **IPENLPPLTA** **DFAEDKDVCK** *NYQEAKDAFL*
*GSFLYEYSR***R** **HPEYAVSVLL** **R***LAK***EYEATL** **EECCAKDDPH** **ACYSTVFDK***L*
*K***HLVDEPQNL** **IKQNCDQFEK** **LGEYGFQNAL** **IVR**YTR**KVPQ** **VSTPTLVEVS**
RSLGKVGTR**C** **CTKPESER***MP* *CTEDYLSLIL* *NR***LCVLHEK**T PVSEKVTK**CC**
TESLVNRRPC **FSALTPDETY** **VPK**AFDEK**LF** **TFHADICTLP** **DTEK**QIK**KQT**
ALVELLK*HK*P K**ATEEQLKTV** **MENFVAFVDK** **CCAADDKEAC** **FAVEGPK***LVV*
STQTALA

Fig. 6. BSA sequence coverage obtained by nanoUHPLC-ESI and LC-MALDI MS analysis of 5 pmol of a tryptic digest using FA and TFA, respectively, as mobile phase additive. Sequences in **bold** were identified in both analyses. Sequences in *italics* were identified only by nanoUHPLC-ESI MS.

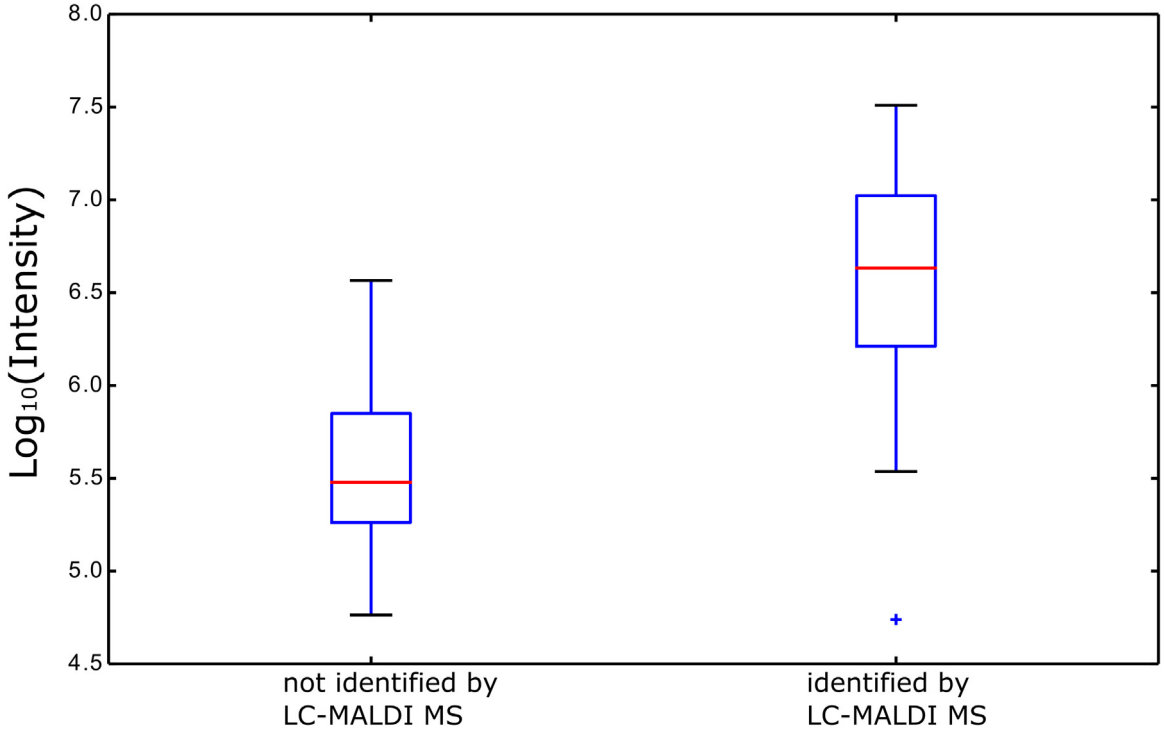

Fig. 7. LC-ESI MS ion signal intensity of the BSA-matched unique peptides separated into two classes according to their detection in a comparative LC-MALDI MS analysis.

led to comparable though slightly lower protein sequence coverages but at vastly lower sample consumption when compared to on-line LC-ESI MS(/MS). In contrast to ESI MS, the usage of TFA as an LC ion pairing reagent does not cause adverse effects in liquid AP-MALDI MS. Furthermore, the MALDI target plate with spotted liquid samples could be stored after analysis for weeks without analyte ion or sample degradation. Further optimization areas for improving the method's current performance, in particular detection limits, have been identified and discussed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijms.2016.12.006.

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